CHARACTERIZATION OF CTP SYNTHASE IN ZEBRAFISH (*Danio rerio*)

by

NURUL WAHIDAH BINTI ABDUL WAHAB

Thesis submitted in fulfilment of the requirements for the degree of Master of Science

August 2019

ACKNOWLEDGEMENT

In the name of Allah, Most Gracious, Most Merciful. Alhamdulillah, all praise to Allah (SWT) who has given me strength, idea, patience and passion throughout this journey. I would like to thank my supervisor, Dr. Mohd Ghows bin Mohd Azzam for the opportunity, guidance, patience, and understanding. Thank you for always believe in me. Special thanks also to my co supervisor Prof. Alexander Chong Shu Chien, for the knowledge on zebrafish. To Cancer Research Malaysia, Shaun Kazuhide Okuda, and Ng Mei Fong, for all the guidance, advices and knowledge. To my labmates and friends that I have come across this journey, all the knowledge we share together and the fun that we have together will always be among my most treasured memories. Last but not least, to my family, mak and bapak, and my siblings for all the support, financially and emotionally. For always be there for me. To my new family, mama daddy especially, thank you for everything. To my other half, my soulmate thank you for the patience and understanding.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ii

TABLE OF CONTENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

LIST OF ABBREVIATIONS ix

ABSTRAK xi

ABSTRACT xiii

CHAPTER 1 INTRODUCTION 1

1.1 Overview 1

1.2 Objectives 3

CHAPTER 2 LITERATURE REVIEW 4

2.1 CTP synthase: An essential metabolic enzyme 4

2.2 Cytoophidium: A novel organelle containing CTP synthase 7

2.3 DON treatment induce formation of cytoophidia 10

2.4 Zebrafish, a powerful model organism 12

2.5 CTP synthase in zebrafish 18

2.6 Morpholino: Zebrafish knockdown method 20

CHAPTER 3 MATERIAL AND METHODS 23

3.1 Zebrafish husbandry 23

3.1.1 Zebrafish water system 23
3.1.2 Feeding 23
3.1.3 Spawning 24
3.1.4 Egg collection 24

3.2 Immunohistochemistry 25

3.2.1 Embryos fixing 25
3.2.2 Phalloidin and hoechst staining 25

3.2.3 Antibody staining ... 26
3.2.4 Mounting and imaging ... 26
3.3 DON treatment ... 27
3.4 Morpholino Oligonucleotides (MO) knockdown ... 27
3.4.1 Morpholino microinjections ... 29
3.4.2 Phenotype observation ... 32
3.5 RNA extraction ... 33
3.6 Agarose gel electrophoresis ... 34
3.7 Zebrafish phylogenetic analysis ... 34
CHAPTER 4 RESULTS ... 35
4.1 Immunohistochemistry staining results ... 35
4.2 CTPS aggregation appear to be cytoplasmic ... 38
4.3 Reduction of CTPS activity by DON treatment ... 39
4.3.1 DON prevent the inflation of zebrafish swimbladder ... 42
4.3.2 DON prevent the inflation of zebrafish swimbladder ... 43
4.4 Morpholino Oligonucleotide results ... 45
4.4.1 One picomolar is sufficient to elicit effect of CTPS gene knockdown in zebrafish ... 47
4.4.2 The *ctps1a* knockdown embryos display more severe effect compare to *ctps1b* knockdown embryos ... 52
4.4.3 Severity of simultaneous CTPS gene knockdown is concentration dependent ... 55
4.4.4 Mortality rates of the knockdown ... 58
4.4.5 Gel electrophoresis shows splice blocking MO successfully reducing the CTPS gene ... 59
4.4.6 The zebrafish phylogenetic analysis ... 61
CHAPTER 5 DISCUSSION ... 63
5.1 CTPS is important for pronephric duct and notochord development ... 63
5.2 Immunostaining correlates with DON treatment and gene knockdown ... 66
5.3 Potential gene dosage between *ctps1a* and *ctps1b* ... 69
5.4 Higher mortality rates on single knockdown ... 74

CHAPTER 6 CONCLUSION AND RECOMMENDATION............................ 76

REFERENCES.. 78

APPENDICES

LIST OF PUBLICATION

LIST OF TABLES

Page

Table 2.1 Taxonomy of zebrafish 13

Table 2.2 Description of each period in zebrafish early developmental stages 15

Table 3.1 List of MO sequence and the primers that was used to validate them 29

Table 4.1 Percentage of knockdown embryos that gives normal phenototype after 3dpf on 1.0pmol 51

Table 4.2 Percentage of knockdown embryos that gives normal and abnormal phenotype after 3dpf on 2.0pmol 51

Table 4.3 Percentage of representative phenotypes of the surviving 1a-kde after 1dpf 52

Table 4.4 Percentage of representative phenotypes of the surviving 1b-kde after 1dpf 54

Table 4.5 Ratio of genes used for simultaneous knockdown 56

LIST OF FIGURES

		Page
Figure 2.1	Synthesis of CTP from UTP	5
Figure 2.2	Cytoophidia formation in fruit flies, yeast and human cells	8
Figure 2.3	Adult WT zebrafish	14
Figure 2.4	In situ hybridization from the previous study on CTPS zebrafish embryos	19
Figure 2.5	Segment of a Morpholino-RNA heteroduplex	21
Figure 3.1	Process of microinjection	31
Figure 3.2	Embryos post-injection	32
Figure 4.1.1	Shows aggregation of CTPS on the early stage of zebrafish embryonic development	36
Figure 4.1.2	Shows aggregation of CTPS on the early larvae stage of zebrafish development	37
Figure 4.1.3	CTPS aggregation on zebrafish	38
Figure 4.1.4	CTPS is cytoplasmic	38
Figure 4.2.1	Preliminary DON treatment on zebrafish after 2dpf	40
Figure 4.2.2	Lateral view of the treated embryos, including control after 6dpf	41
Figure 4.2.3	Position of swimbladder on zebrafish larvae during 6dpf	42
Figure 4.2.4	Graph of DON concentration (µg/ml) against body length (mm) of the zebrafish	44
Figure 4.3.1	Zebrafish embryos	46

Figure 4.3.2 Knockdown of each CTPS genes on different concentration after 1dpf 49

Figure 4.3.3 Knockdown of each CTPS genes on different concentration after 3dpf 50

Figure 4.3.4 Representative for phenotype with the highest frequency after 1dpf 52

Figure 4.3.5 Shows representative for phenotype of 1b-kde with the highest frequency after 1dpf 54

Figure 4.3.6 Shows embryos from their respective gene knockdown including ControlMO on 2dpf and 3dpf 55

Figure 4.3.7 Double knockdown embryos after 1dpf and 2dpf 57

Figure 4.3.8 Graph of mortality rates (%) for each knockdown groups 59

Figure 4.3.9 . PCR confirms knockout of either CTPS genes 60

Figure 4.4.1 CTPS duplication in zebrafish resulted in two highly similar genes 62

Figure 5.1 Accumulation of CTPS that occur on pronephric duct 64

Figure 5.2 Location of the CTPS accumulation on the notochord 65

Figure 5.3 Shows 1b-kde on 3dpf and TEST2 knockdown embryos on 2dpf 68

Figure 5.4 Flowchart on how splice blocking MO trigger NMD 70

Figure 5.5 TEST3 knockdown embryos with embryos treated with 1000μg/ml concentration of DON 72

LIST OF ABBREVIATIONS

µg /ml	Microgram per mililiter
µL	Micro liter
µm	Micro meter
ATP	Adenosine-5'-triphosphate
cDNA	Complimentary DNA
CRISPR/cas	Clustered regularly interspaced palindromic repeats/CRISPR associated
CTP	Cytidine-5'-triphosphate
CTPS	CTP synthase
CTP*syn*	CTP synthase
DNA	Deoxyribonucleic acid
DON	6-diazo-5-oxo-L-nor-Leucine
Dpf	Day post fertilization
GTP	Guanosine-5'-triphosphate
Hpf	Hour post fertilization
miRNA	microRNA
mm	Mili meter
MO	Morpholino oligonucleotides
mRNA	Messenger RNA
NGS	Natural goat serum
nl	Nano liter
NMD	Nonsense mediated decay
NTP	Nucleotide triphosphate
PBS/PBT	Phosphate buffer saline/plus Triton-X
PFA	Paraformaldehyde

pmol	Picomolar
RBP	RNA-binding protein
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotation per minute
SSD	Smaller scale duplication
*tg*CTPS	*Toxoplasma gondii* CTPS
UTP	Uridine-5'-triphosphate
WGD	Whole genome duplication

PENCIRIAN CTP SINTASE DALAM IKAN ZEBRA (*DANIO RERIO*)

ABSTRAK

CTP sintase (CTPS) adalah enzim yang memangkin pengeluaran sitidina trifosfat, iaitu salah satu daripada empat nukleotida trifosfat yang membentuk DNA dan RNA. Ketidakseimbangan CTP memberi implikasi di dalam pelbagai penyakit dan kanser. Kajian sebelum ini telah menunjukkan CTPS boleh membentuk struktur berfilamen yang dipanggil cytoophidia. Struktur ini ditemui daripada bakteria sehingga manusia. Namun, tujuan di sebalik pembentukkan cytoophidia ini masih belum difahami sepenuhnya. Sehingga kini, belum ada kajian mendalam mengenai CTPS pada *Danio rerio* (ikan zebra) dan terdapatnya dua CTPS gen di dalam ikan zebra, menggunakan ikan zebra sebagai model organisma boleh membantu untuk memahami dengan lebih lanjut tentang CTPS dan cytoophidia. Pewarnaan antibodi ke atas embrio ikan zebra menunjukkan CTPS tertumpu pada kawasan duktus pronefros dan notokord di hujung ekor. Pengesahan data selanjutnya dilakukan dengan membuat rawatan drug 6-diazo-5-oxo-l-norleucine (DON) terhadap embrio ikan zebra untuk melihat bagaimana pengekspresan lebih CTPS memberi kesan kepada pertumbuhan awal ikan zebra kerana DON diketahui untuk menggalakkan pembentukkan cytoophidia dalam yis, lalat buah dan sel manusia. Rawatan DON pada kadar kepekatan yang berbeza memberi kesan keterukan yang berbeza dan kegagalan untuk mengembungkan pundi renang didapati pada semua ikan zebra yang dirawat dengan DON. Seterusnya, Morpholino oligonukleotida (MO), salah satu cara menyahfungsi gen untuk memerhati kesan pengurangan fungsi CTPS pada awal pertumbuhan ikan zebra, digunakan. Dua MO telah di reka terhadap dua isoform CTPS di dalam ikan zebra iaitu *ctps1a* dan *ctps1b* melalui cara mikrosuntikan.

Perbandingan di antara fenotip nyahfungsi gen *ctps1a* dan nyahfungsi *ctps1b* pada 24hpf (jam selepas persenyawaan) dan 48hpf menunjukkan sementara nyahfungsi gen *ctps1a* mengganggu pertumbuhan dengan serius, embrio yang dinyahfungsi gen *ctps1b*, membesar pada kadar yang normal,tidak memiliki fenotip yang luar biasa sehingga pada 3dpf (hari selepas persenyawaan), beberapa embrio menunjukkan fenotip bengkok pada hujung ekor. Namun, apabila kedua dua gen *ctps1a* dan *ctps1b* dinyahfungsi serentak, berkemungkinan kedua dua gen bergantung antara satu sama lain. Eksperimen ini mampu memberi pemahaman awal tentang bagaimana penyingkiran CTPS menggunakan nyahfungsi gen dan rawatan drug DON menjejaskan fenotip ikan zebra pada awal pembesaran serta menjadi garis panduan untuk melakukan penyiasatan terhadap pencirian CTPS pada ikan zebra dengan lebih mendalam.

CHARACTERIZATION OF CTP SYNTHASE IN ZEBRAFISH

(*Danio rerio*)

ABSTRACT

CTPS is an enzyme that catalyzes the synthesis of cytidine triphosphate (CTP), one of the four basic nucleotide triphosphates that make up DNA and RNA. CTPS dysregulation has been implicated in many different diseases and cancers. It was also shown previously that CTPS can form filamentous structures called cytoophidia. This structure was found on yeast, fruit flies and human. Nevertheless the mechanism behind cytoophidia formation is still vague. To date, there are no in depth studies yet on characterizing CTPS on Danio rerio (zebrafish) and as there are two CTPS genes in zebrafish i.e *ctps1a* and *ctps1b* utilizing zebrafish as model organism can further help to understand the CTPS and its cytoophidia. In this studies, the antibody staining results shows that in developing embryos, CTPS is concentrated on the pronephric duct area and the notochord at the end tail area. Next treating embryos with 6-diazo-5-l-norleucine (DON), was DON to observe how overexpression of CTPS will affect zebrafish embryos during its early development as DON was known to promote cytoophidia in yeast, fruit flies and human cells. Different dosage showed differences in severity of the phenotypes and failure to inflate swimbladder was observed on all treated embryos. Morpholino oligonucleotides (MO), a tool for gene knockdown was next used to observe how reducing the CTPS functions will affect zebrafish embryos during its early developmental stage. Two MO were designed against the two CTPS genes in zebrafish and the delivery of the MO was done through microinjection. Comparison between the phenotypes of *ctps1a* and *ctps1b* knockdown in 24hpf (hour post

fertilization) and 48hpf embryos revealed that whilst elimination of *ctps1a* severely impaired growth, *ctps1b* deficient embryos, did not exhibit any unusual phenotype until 3dpf where some of the embryos then developed slight bent on the end tail. However, when both *ctps1a* and *ctps1b* are knockdown simultaneously, it reveals that both genes are co-dependent on each other. These experiments can provide an initial insight on how the elimination of CTPS via knockdown and drug treatment affect the phenotype of zebrafish on its early development hence can act as guidelines for further investigation on the characterization of CTPS in zebrafish.

CHAPTER 1
INTRODUCTION

1.1 Overview

Metabolic enzymes are essential tools that carry out variety of cellular functions necessary for survival and homeostasis, including cellular respiration, energy storage and transcription (Hsu and Sabatini, 2008). All of these pathways are crucial for development and maintenance of cell integrity. Dysregulation of metabolic pathways are known as roots for many diseases and this has raised interest in the studies that involve targeting metabolic enzymes (Rosario *et al.*, 2018).

ATP synthase is one of the most studied metabolic enzyme that is known to be vital for cell growth and development. It is an enzyme that creates the energy storage molecule adenosine triphosphate (ATP) and is located in the inner membrane of mitochondria. Alteration of ATP synthase biogenesis may cause two types of isolated defects, qualitative when the enzyme is structurally modified and does not function properly, and quantitative when it is present in insufficient amounts (Houštěk *et al.*, 2006). Dysregulation of ATP synthase in human results in various diseases proving that it is an essential component needed in cell growth and development.

Similarly, CTP Synthase (CTPS) is an enzyme that generates cytidine-5-triphosphate (CTP) which is needed for the formation of DNA and RNA. The CTP not only plays a role in building blocks that make up RNA and DNA but it also have roles in other additional biological processes for example phospholipid synthesis and protein sialylation (Higgins *et al.*, 2007). Compared to ATP not much is known about CTP and its role in the cellular function mechanism.

CTPS is abundance in a filamentous structure called cytoophidium (cellular serpent in Greek) (Liu, 2010). Little is known as to why they form the serpent like structure and it is intriguing to learn what role does it play as according to another recent studies, the structure is also found in bacteria (Ingerson-Mahar *et al.*, 2010), budding yeast, rats and also human (Chen *et al.*, 2011), showing that the structure is highly conserved and indispensable throughout evolution.

As the studies on CTPS on bacteria, fruit flies and human cells have been conducted, there is no studies have been done on zebrafish (*Danio rerio*) yet. This model organism have many advantages that can be utilized such as the embryos are transparent that they can be observed using simple dissecting microscope and the embryos are known to be robust that they are able to survive different procedure right after fertilization, including genetic manipulation, morpholino or ribonucleoprotein (CRISPR/Cas) microinjection at single cell stage, as well as cancer cell xenotransplants (Gutiérrez-Lovera, 2017).

Here, characterizing the role of CTPS in zebrafish will be done and given that there are 2 copies of CTPS gene in zebrafish namely *ctps1a* and *ctps1b* thus it is hypothesized that CTPS plays an important role in zebrafish development.

1.2 Objectives

The objectives of this research are:

i. To characterize the spatial and temporal expression of CTPS in zebrafish

ii. To identify the effect of dysregulation of CTPS in zebrafish through DON treatment

iii. To elucidate the function of CTPS during zebrafish development through MO knockdown.

1.2 Objectives

The objectives of this research are:

i. To characterize the spatial and temporal expression of CTPS in zebrafish

ii. To identify the effect of dysregulation of CTPS in zebrafish through DON treatment

iii. To elucidate the function of CTPS during zebrafish development through MO knockdown.

CHAPTER 2
LITERATURE REVIEW

2.1 CTP synthase: An essential metabolic enzyme

CTP Synthase (CTPS) is an enzyme that produce cytidine-5'-triphosphate (CTP) one of the basic nucleotide triphosphates (NTPs) that make up RNA alongside with adenosine-5'-triphosphate (ATP), uridine-5'-triphosphate (UTP) and guanosine-5'-triphosphate (GTP). All of the NTPs have their own essential roles in cell development and function.

UTP is transformed into CTP by the replacement of a carbonyl group by an amino group. The reactions requires ATP and uses glutaminase as the source of amino group (**Figure 2.1**). The reaction then go through an analogous mechanism in which the O-4 atom is phosphorylated to form a reactive intermediate, and then the phosphate is displaced by ammonia, freed from glutamine by hydrolysis (Azzam and Liu, 2013).

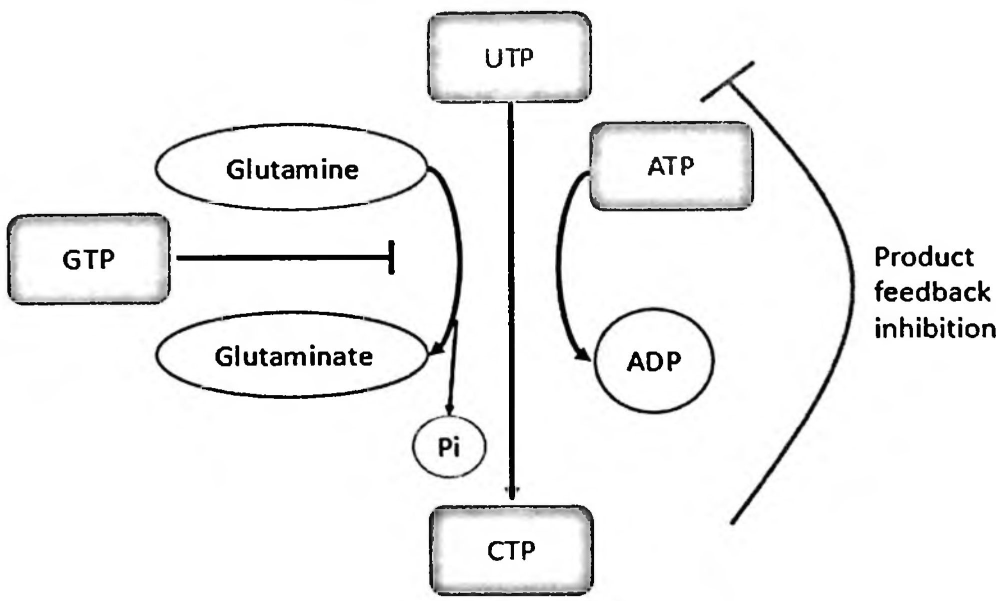


Figure 2.1. Synthesis of CTP from UTP. CTPS produces CTP from UTP and glutamine which regulates intracellular CTP levels through interactions with all four nucleotides triphosphates.

CTP is generated from UTP in three reaction steps (kinase reacton, glutaminase reaction and ligase reaction) involving two active sites (Levitzki & Koshland, 1971). The glutamine amide transfer domain catalyzes the conversion of glutamine to glutamate and NH_3 and requires GTP for allosteric activation. Amidotransferase activity produced ammonia that participates in the ATP-dependant conversion of UTP to CTP that then carried out by the synthetase domain. CTPS then carries out the terminal reaction in the *de novo* synthesis of pyrimidine nucleotides that eventually accommodate precursor for DNA, RNA, phospholipids and sialoglycoproteins (Robertson, 1995).

The CTP product provides a negative feedback by competitively inhibiting the UTP substrate in a way the uracil O-4 phosphatase atom that was displaced by ammonia to yield CTP product, serve as negative feedback by competitively inhibit

UTP substrate (Aronow & Ullman, 1985). CTPS present as dimer which aggregates into tetramer based on its concentration and other factors (Anderson, 1983).

CTPS activity is not only important for producing building blocks for RNA, DNA and sialoglycoprotein production but is also important for phospholipids production (Yang and Carman, 1996). Phospholipids are class of lipids that are a vital part of cells components. This enzyme plays an essential role in the synthesis of all membrane phospholipids in eukaryotic cells, in a way that its reaction product, CTP, is the direct precursor of the activated, energy rich phospholipid pathway intermediates (Ostrander *et al.*, 1998). In the yeast *Saccharomyces cerevisiae,* the reaction product of enzyme CTPS encoded by the name *URA7* and *URA8*, is an essential precursor of all membrane phospholipids that are synthesized via the Kennedy and CDP-diacylglycerol pathways. Mutations that alleviate this regulation result in an elevated cellular level of CTP and an increase in phospholipid synthesis via the Kennedy pathway (Wilcox *et al.*, 2014).

Likewise, in humans CTP is generated by enzyme CTPS encoded by the name *CTPS1* and *CTPS2*, where its activity in humans hold a potentially significant step for DNA synthesis in lymphocytes. The loss of function homozygous mutation in *CTPS1* in humans that causes a novel and life-threatening immunodeficiency, characterized by an impaired capacity of activated T and B cells to proliferate in response to antigen receptor-mediated activation (Martin *et al.*, 2014). According to the same study, *CTPS1* may represent a therapeutic target of immunosuppressive drugs that could specifically dampen lymphocyte activation.

Growing interest in CTPS have provide substantial new finding as it was discovered lately in a studies using fruit flies that optic lobes development are

delayed in *CTPsyn* mutants and *CTPsyn* RNAi (Tastan & Liu, 2015). Overexpressing CTPS impairs the development of optic lobes, specifically by blocking the transition from neuroepithelium to neuroblast (Tastan & Liu, 2015). On the whole, the studies have come to the conclusion that CTPS is essential for optic lobe homeostasis in fruit flies.

In recent times, it was discovered that CTPS can serve as a potent drug target for *Toxoplasma gondii* (Narvaez-ortiz *et al.*, 2018) where, unlike human host cells, these parasites cells cannot compensate for the lack of CTPS activity. An analogous phenotype was observed when the parasite was under nutrient stress it disrupts the lytic cycle and the tgCTPS (*Toxoplasma gondii* CTPS) was refractory to a genetic deletion, insinuate an essential requirement of this enzyme for the parasite.

In mammalian cells, inhibition of CTPS plays an important role in the balance of the pyrimidine nucleoside triphosphate pools (Ostrandor *et al.*, 1998). Furthermore, CTPS is an attractive target for cancer therapy and also drug development against viral and parasitic disease (e.g African sleeping sickness, malaria, and infectious blindness). The enzyme CTPS is also known to be upregulated in many forms of cancer such as leukemia, hepatomas and colon cancer (Liu, 2011). Conjointly, it is no surprise as to why there are growing interest to study more on CTPS.

2.2 Cytophidium: a novel organelle containing CTP synthase

The cytoophidium (plural: cytoophidia) (Liu, 2010) is a filamentous structures that is formed from CTP synthase protein. The occurrence of the structure was discovered independently by different groups of researcher where it exist in a range of organisms that is bacteria (Ingerson-Mahar *et al.*, 2010), budding yeast and

rats (Noree *et al.*, 2010) and fruit flies (Liu, 2010). Making it more interesting, another study also discovered that CTPS also forms cytoophidium in human cells (Chen *et al.*, 2011). This shows that cytoophidia is highly conserved in a wide range of organisms, across prokaryotes and eukaryotes (Liu, 2011) **(Figure 2.2).**

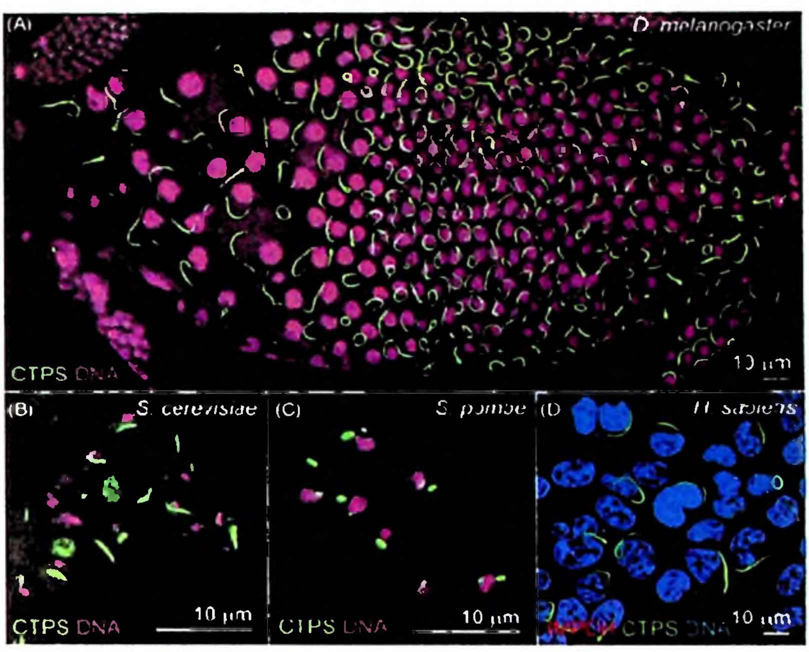


Figure 2.2. Cytoophidia formation in fruit flies, yeast and human cells. Image of cytoophidia (green) in *D. melanogaster, S. cerevisiae, S. pombe* and *H. Sapiens.* (Aughey & Liu, 2016)

CTP synthase can form cytoophidia not only in the cytoplasm, but the enzyme can also form cytoophidia in the nucleus of eukaryotic cells (Gou *et al.*, 2014). Previous study shows that cytoophidium regulates cell shape in a curved bacterium, *Caulobacter cresentus* (Ingerson-Mahar *et al.*, 2010) the exact function of this novel subcellular structure in other organism is still ambiguous.

Cytoophidium formation can be induced either by inhibition or overexpression where cytoophidia could be induced to form when human cells and fruit flies tissues were treated with the glutamine analogues 6-diazo-5-oxo-l-norleucine (DON) and azaserine, where both drugs act as competitive inhibitors of CTP synthase (Aughey *et al.*, 2014).

It was discovered by (Barry *et al.*, 2014) that CTP synthase is almost completely inactivated when the enzyme forms polymers. CTP encourages the polymers to form, whilst UTP and ATP cause them to disassemble. This means that the enzyme is less active when there is excess product in the cell, and most active when its substrate is plentiful (Barry *et al.*, 2014). They also conclude that polymerized CTP synthase enzymes are inactive and must disassociate from the polymer to resume normal enzymatic activity. Another study suggest that one of the possible function of cytoophidia is to form a centralized 'depot' of CTPS that allows for faster re-activation of the enzyme following periods of low nutrient availability to quickly increase the intracellular CTP pool (Aughey & Liu, 2016).

A different research group discovered that cytoophidia formation was a response towards nutrients distress where membraneless organelles allow rapid phase transition through the assembly and disassembly of macromolecules in response to environmental cues (Lin *et al.*, 2018). The study also demonstrated that histidine donates one carbon to the methionine cycle through cystolic folate cycle, which is required for cytoophidia assembly during glutamine deficiency and nutrient deprivation. They found that CTPS is preserved by forming filaments, which is a strategy for maintaining homoestasis during starvation.

The observation that CTPS may localize to different membrane bound cellular compartments invites the speculation that filament formation could act to prevent the movement of CTPS into or out of the nucleus as required. Cytoophidia therefore may represent a novel mechanism to spatially regulate enzyme activity in addition to its role in downregulating catalysis (Aughey & Liu, 2016).

The aptness of CTP synthase to form such structure makes it an ideal model system for further understanding on how the mechanism of enzyme activity that able to assemble into visible supramolecular complexes works (Noree *et al.*, 2014). The high conservation and widespread distribution of the cytoophidium among diverse organisms and cell-types indicates that this novel compartment contribute to fundamental cellular processes. Cytoophidium does not only possess considerable importance in the cell and developmental biology fields, but also relevant to the understanding of the pathological mechanism of human diseases.

2.3 DON treatment induce formation of cytoophidia

It is proven that DON (6-Diazo-5-oxo-L-norleucine) treatment could promote cytoophidium assembly in various types of tissues in *Drosophila melanogaster*, including larval testes, ovaries, salivary glands, guts, trachea, adult testes and brain (Chen *et al.*, 2011). Observing the effect of DON on CTPS in the previous studies on *Drosophila* and human cells (Chen *et al.*, 2011), it is interesting to study the effect of DON on zebrafish as it is known to be associated with CTPS. DON is a glutamine analog that has been used as an inhibitor of glutamine utilizing enzymes for example, carbomoyl phosphate synthase (CAD), CTP synthase, FGAR amidotransferase, guanosine monophosphate synthetase (GMPS), and glutaminase. DON was initially delineate as an antitumor antibiotic isolated from unidentified streptomyces strain (Cervantes-Madrid *et al.*, 2015).

Biochemical studies on DON reveal there is a two step, mechanism-based mode of inhibition across multiple glutamine-using enzymes (Tenora *et al.*, 2019). First, DON binds competitively to the glutamine active site, after that a covalent adduct is formed irreversibly inhibiting the enzyme. DON act as a selective

mechanism-based inactivator of glutamine-using reactions rather than a non-specific reactive intermediate (Tenora *et al.*, 2019). DON inhibits glutamine-using enzymes including glutaminase at low micromolar levels as well as multiple glutamine amidotransferases invloved in *de novo* purine and purimidine synthesis and hexosamine production (Tenora *et al.*, 2019).

The kinetics of inhibition and inactivation have been described for some, though not all, of DON's target enzymes. At far higher concentrations DON also serves as a substrate and an inhibitor of several amino acid transporters and transglutaminases as well as a number of amino acid synthesis reactions more relevant in prokaryotic systems (Tenora *et al.*, 2019). DON inhibited the growth of multiple cancer cell lines in culture, prevented tumor growth , and increased survival in several murine cancer models including sarcomas, carcinomas, and leukemias in early preclinical cancer models. It is also worth to note that the most effective dosing regimens in some early rodent studies were daily low-dose therapy (Tenora *et al.*, 2019).

Treating *Drosophila melanogaster* with DON on larval stage have shown profound effect on larval development. Low doses of DON delayed development, while higher dosages halted the growth of larval at the first instar (Chen *et al.*, 2011). This was probably due to the fact that DON, as a glutamine analog, influence several glutamine-dependant metabolic enzymes including CTP synthase. The constant presence of DON as a replacement for L-glutamine ultimately lowers cellular levels of the α-amino acid (Ahluwalia *et al.*, 1990). As L-glutamine is the primary nitrogen source for CTPS-catalysed reactions, its limited availability eventually inactivates the protein.

DON also stimulate *Tg*CTPS assembly in a concentration-dependant manner causing an increase in the occurance of filament-like structure formation and appears to be promoted not only subsequet to the release of tachyzoites from the host cell but also by DON (Narvaez-Ortiz *et al.,* 2018). Tachyzoites is a rapidly multiplying stage in the development of the tissue phase of infected tissues. DON treatment of *T. gondii* tachyzoites resulted in morphologically altered parasites where the loss of the typical rodette organization was observed in intracellular parasites and the parasitophorous vacuole of the aberrant parasites were amorphous with a large vacuolar space (Narvaez-Ortiz *et al.,* 2018).

It is hypothesized that treating zebrafish with DON will also affect zebrafish development and morphology as it did with other eukaryotic CTPS in previous studies on human, Drosophilla, and mammalian cells (Narvaez-Ortiz *et al.,* 2018). Treating zebrafish with DON will help to understand how dysregulation of CTPS in terms of overexpression.

2.4 Zebrafish, a powerful model organism

Danio rerio or commonly known as zebrafish are a common freshwater aquarium fish that, over the last 40 years have gain interests among researchers that have proven its ability to become a powerful model organism for research into various biology fields (Meyers, 2018). Zebrafish's natural environment was observed in a slow moving or still waters in floodplains in the state of Bihar at the rivers of northeastern India (Engeszer *et al.*, 2007). Zebrafish is a bony fish (teleost) that belongs to the family Cyprinidae under the class of Actinopterygii (ray-finned fishes) (Rahman Khan & Sulaiman alhewairini, 2018). The taxonomy descriptions of zebrafish are listed in the **(Table 2.1)**.

Table 2.1 Taxonomy of zebrafish.

Zebrafish	
Kingdom	Animalia
Phylum	Chordata
Class	Teleostei
Order	Cypriniformes
Family	Cyprinidae
Genus	Danio
Species	*Danio rerio*

The small teleost fish can easily being recognized by its pigmented, horizontal blue stripes, 5 to be exact on the side of the body which extend to the end of the caudal fin (Spence *et al.*, 2007). These stripes resemble the zebra's stripes which give rise to its common name, zebrafish. Adult zebrafish **(Figure 2.3)** size rarely exceeds 40 mm in length with a fusiform body shape and laterally compressed, with a terminal oblique mouth directed upwards. It is difficult to differentiate between juvenile male and female as both sexes looks almost the same. However entering adult phase, male zebrafish will have a more slender body shape while the female possess larger and rounder belly.

Figure 2.3. Adult zebrafish. Lateral view of adult female (top) and male (bottom) zebrafish (Murakami, 2014).

Zebrafish early life cycle comprises of eight periods that is zygote, cleavage, blastula, gastrula, segmentation, pharyngula, hatching, and early larvae. Each period covers different early developmental point as describe in **Table 2.2** according to (Meyers, 2018).

Table 2.2. Description of each period in zebrafish early developmental stages.

Period	Hpf (hour post fertilization)	Description
Zygote	**0**	The newly fertilized egg through the first zygotic cell cycle.
Cleavage	**0.75**	Early mitotic cell division occuring in the blastodisc, where the development encompassing the first six ygotic cell cycles.
Blastula	**2.2**	Cleavages produces a mass of cells at the animal pole of developing embryo, the period of development when the blastodisc begins to look ball-like at the 128-cell stage through the time of onset gastrulation.
Gastrula	**5.25**	Period of development beginning at the onset of involution that produces the two primary germ layers, the epiblast and hypoblast and during which the definitive embryonic axis forms by convergence and etension movements.
Segmentation	**10**	The period of development between gastrula and pharyngula where epiboly is complete, tailbud and head starts to form. Primary organogenesis and neurulation begins.
Pharyngula	**24**	Developed to the phylotypic stage, body axis straightens, circulation, pigmentation and fins begin development.
Hatching	**48**	Completion of rapid morphogenesis of primary organ systems, hatching occurs.
Early larva	**72**	Swimbaldder inflates, food-seeking and active avoidance behaviour.

One of the important quality of zebrafish is the availability of a large growing array of molecular information (Scott & Stemple, 2004). The existence of plethora of genetic and experimental manipulations of the current established laboratory methods, which include methods of obtaining haploid and homozygous fish, techniques for primary cell cultures, and techniques for transient stable transgenesis (Babin *et al.*, 2014).

Another advantages of this organism is the extraordinary range of optical methods possible in the developing embryo and larval fish (Parkin *et al.*, 2009). The ex vivo development of transparent zebrafish embryos allows the direct and dynamic observation of cellular processes. There is a significant degree of transparency up to 5 days post fertilization. Individual living cells and even cellular organelles can be observed directly with no staining and little preparation of the fish and no tools other than a good microscope. With the use of optical sectioning techniques, it is possible to visualize many structures, even subcellular organelles, with exceptional clarity.

Zebrafish is a model organism of vertebrate that is receiving increasing attention as a model of human disease and drug discovery. Detailed automatic and manual annotation provides evidence of more than 26,000 protein-coding genes, the largest gene set of any vertebrate so far sequenced. Comparison to the human reference genome shows that 71.4% of human genes have at least one zebrafish orthologue as defined by Ensembl Compara. Concurrently, 69% of zebrafish genes have at least one human orthologue (Howe *et al.*, 2013). Among the orthologue genes, 47% of human genes have one-to-one relationship with a zebrafish orthologue (Howe *et al.*, 2013). Sharing a high level of genetic and physiologic homology with humans, which includes brain, digestive tract, musculature, vasculature, and an

innate immune system (Zhao *et al.*,2015), making them a good model organism to study.

Zebrafish also has been used as a model for carcinogen effects and development of cancer studies. The genes regulating cell cycle, cell proliferation, and apoptosis have already been screened in yeast, fruitflies and *C. elegans*, in the similar way gene functions for these biological pathways can be screened in zebrafish to understand the events that lead to the development of cancer in any vertebrate species (Amatruda *et al.*, 2002).

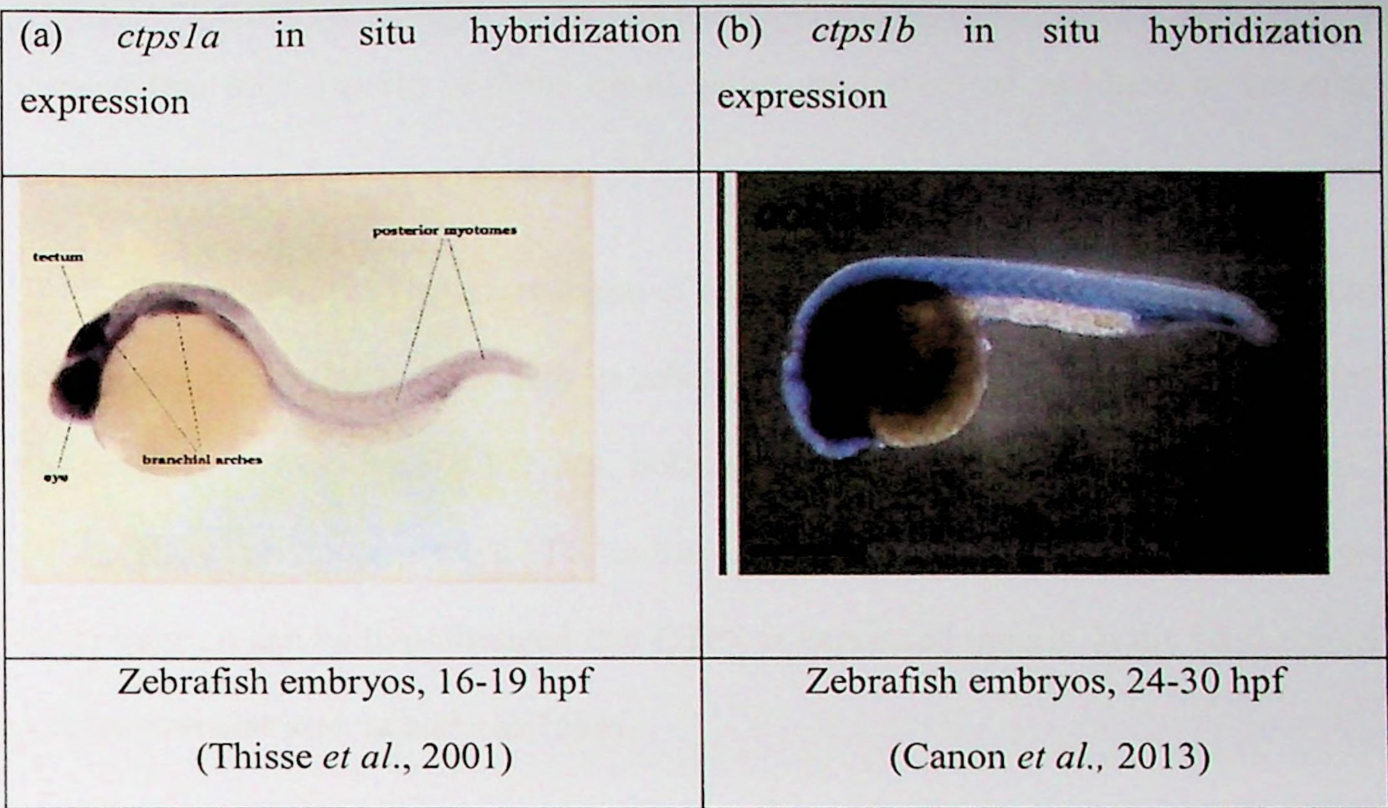
In developmental biology, zebrafish has been used to see the development of various systems such as development of the enteric nervous system, angiogenesis and regeneration (Rahman Khan & Sulaiman alhewairini, 2018). Highly conserved cancer pathways can be screened genetically using zebrafish although, primarily cancer is a disease of adults, but through mutagenesis screens, cell cycle phenotype could be examined in rapidly developing transparent embryos of zebrafish (Rahman Khan & Sulaiman alhewairini, 2018).

The past few years have witness rapid new emerging tools such as CRISPR and zinc finger nucleases that allows effective gene knockout in zebrafish. Brainbow and MAZe are the example of transgenic technologies that have made it possible to analyse the convolution of the developmental processes and gain insights into the mechanisms of diseases in a vertebrate. Brainbow is a genetic cell-labelling technique where hundreds of different hues can be produced by stochastic and combinatorial expression of a few spectrally different fluorescent proteins (Weissman & Pan, 2015).

On the other hand, MAZe (mosaic analysis in zebrafish), serve way to trace cell lineages in a developing vertebrate and also a way to observe, in vivo, how behavior of individual cells are affected by the genes they express (Collins *et al.*, 2010). The emergence of tools to ease the study on this model organism proved that zebrafish is a valuable model organism that is sought after researchers that have interest to study on vertebrates. Consequently, utilizing zebrafish as a vertebrate model organism, would help to comprehend the CTPS and its cytoophidia better.

2.5 CTP Synthase in zebrafish

At present, there are no in-depth study yet have been conducted on characterizing the function of CTPS in zebrafish. In situ hybridization **(Figure 2.4)** screen have been conducted by Thisse *et al.*, in 2001 and Cannon *et al.*, in 2013, gives an idea as to where CTPS is expressed in zebrafish. In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA, RNA or modified nucleic acids strand to localize specific DNA or RNA sequence in section or portion of tissue. The *ctps1a* is expressed in the midbrain, pharyngeal arch area, and immature eye during 14-19 somites stage (16-19 hpf). The *ctps1b* on the other hand was expressed on the blood cell and vascular endothelium during prim-5 (24-30 hpf).



(a) *ctps1a* in situ hybridization expression	(b) *ctps1b* in situ hybridization expression
Zebrafish embryos, 16-19 hpf (Thisse *et al.*, 2001)	Zebrafish embryos, 24-30 hpf (Canon *et al.*, 2013)

Figure 2.4. In situ hybridization from the previous study on CTPS zebrafish embryos. (a) The *ctps1a* expression was mainly found on the head area and is associated with genes that is essential during early developmental stages. (b) The *ctps1b* expression was mainly on the head area and pronephric duct area and is associated with genes that is responsible for developing blood and endothelium.

Thisse *et al.*, perform a large scale in situ hybridization screen to characterize genes expressed in a spatially regulated manner during zebrafish embryogenesis. The studies provides hundreds of specific cell tissue markers to analyze mutant phenotypes and to help identify candidates for mutant loci or downstream targets of regulatory genes. The studies also allows the description of zebrafish embryonic development in terms of gene expression and will eveantually establish a 'molecular anatomy' of the developing embryo (Thisse *et al.*, 2001).

Cannon *et al.*, use zebrafish embryos to characterise the transciptome of the developing blood and endothelium, two cell types that closely associated during development. High-throughput sequencing identified 754 genes whose transcripts are enriched three-fold or more in blood and/or vascular endothelial cells compared with the rest of the embryo at 26-28hpf (Canon *et al.*, 2013). Of these genes, 388 were

classified as novel to these cell types after cross-reference with PubMed and the zebrafish information (ZFIN). Analysis by quantitative PCR and in situ hybridisation showed that 83% (n=41) of these novel genes are expressed in blood or vascular endothelium.

The studies provide a catalogue of genes whose expression is enriched in the developing blood and endothelium in zebrafish, many of which will be required for the development of those cell types, both in fish and in mammals (Canon *et al.*, 2013). The expression of the CTPS on the earlier stage, is however unspecified yet. From these, it can be hypothesized that CTPS is expressed mainly in the head region and the vascular area as early as 16hpf.

2.6 Morpholino: Zebrafish knockdown method

Morpholino oligonuleotides (MO) is a tools for blocking sites on RNA to obstruct cellular processes by specifically binding to its selected target site to block access of cell components to that target site. The binding needs 25-base morpholinos for antisense gene inhibition and the backbone makes MO resistant to digestion by nucleases. Each morpholinos consists of a nucleic acid base, a morpholine ring, and a non-ionic phosphordiamidate intersubunit linkage (**Figure 2.5**). It is a nonionic DNA analogs that carry altered backbone linkages compared to RNA or DNA. Although having an altered backbone, MO bind to complementary nucleic acid sequences by Watson-Crick base pairing. As the backbone lacks negative charge, it is believed that MO are less likely to interact non-selectively with cellular proteins (Corey & Abrams, 2001).

About 80% of the MO designed and produced by GENE TOOLS, LLC are effective (typically achieving about 70% to 98% knockdown of the expression of

their intended ptargets). This could be due to their extended length and the inherent high affinity of MO for complementary RNA sequences (Summerton, 2007). MO can only be ordered exclusively from GENE TOOLS, LLC

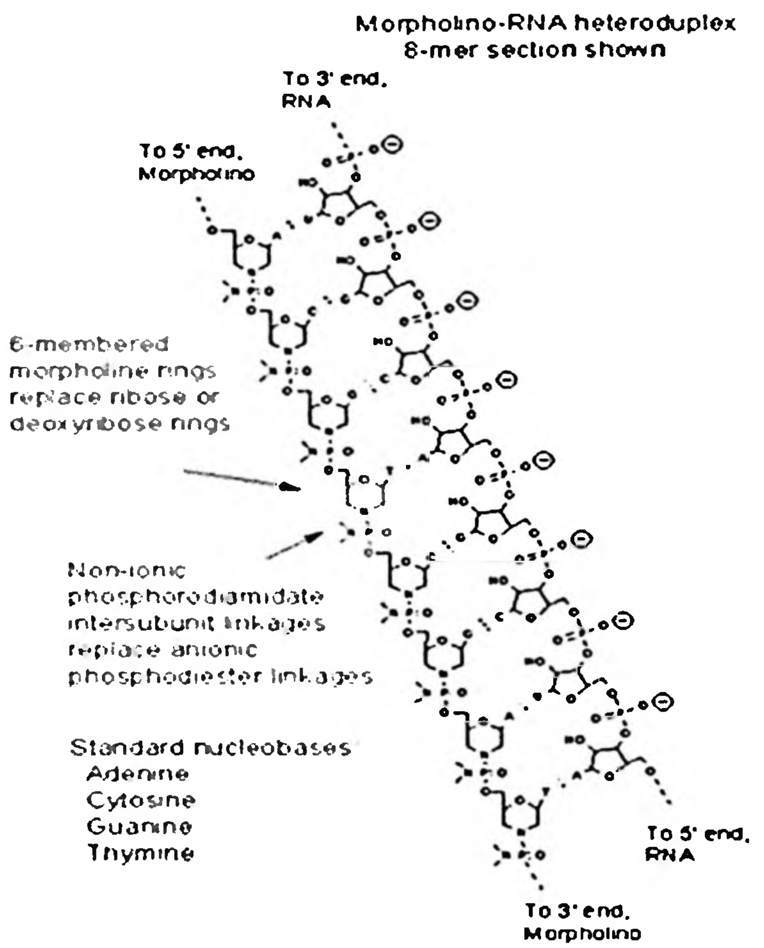


Figure 2.5. Segment of a Morpholino-RNA heteroduplex (Gene Tools LLC., 2007 by Jon D. Moulton)

There are two types of blocking by MO, one is translation blocking and the other one is splice blocking. Translation blocking works by sterically blocking the translation initiation complex (Morcos, 2007). This is enough to knockdown expression of many target sequences entirely enough that after waiting for existing protein to degrade, the target protein band disappears completely from Western Blot. MO generally doesn't cause degradation of their RNA targets, rather, they hinder the biological activity of the target RNA until that RNA is degraded naturally.

Splice blocking on the other hand, works by blocking sites that involves in the pre-mRNA splicing, where it can be used to modify and control normal splicing

events (Morcos, 2007). This event can be validated by RT-PCR. The successful splice- modification will appear as the changes in the RT-PCR product band on an electrophoretic gel. The band might change into a new mass or, if they manage to cause nonsense mediated decay of the transcript, the band will lose its intensity or disappear.

MO is delivered into zebrafish via microinjection during one cell stage or up to 4 cell stage. A control should be included when conducting experiment involving MO to properly analyse the phenotypes. Both MO and its standard control MO can be obtained from Gene Tools, LLC.

CHAPTER 3
MATERIALS AND METHODS

3.1 Zebrafish husbandry

3.1.1 Zebrafish water system

Zebrafish (*Danio rerio*) AB line was used throughout this study. All procedures have obtained approval from the Animal Ethics committee, Universiti Sains Malaysia (205/95/630), and performed in accordance with relevant guidelines and regulations.

Zebrafish is kept in a water system with constant temperature more or less 28.5°C (Linbo, 2009). Adult zebrafish is separated according to its gender to avoid pre mature spawning. The water system is kept in a room with 14/10 hour light/dark cycle (Linbo, 2009). Keeping the temperature and light/dark cycle constant is important to ensure that the fishes comfortable and in optimum condition. The fish tank in the water system was cleaned and check at least once a week to avoid any contamination and to quarantine sick fishes if any.

3.1.2 Feeding

Zebrafish is fed twice a day every day, once in the morning and once in the evening (Jeanray *et al.*, 2015). Overfeeding need to be avoided as it will lead to water contamination. Zebrafish is best feed with brine shrimp or can be alternated with commercial fish food (Gebauer *et al.*, 2011). To keep a healthy adult zebrafish fit for mating, the best diet is to feed them with brine shrimp only twice a day. Brine shrimp is usually prepared a day before every day as it usually takes them overnight to hatch.

3.1.3 Spawning

Before spawning the male and female will be chosen and inserted inside breeding tank with separator between them overnight (in the dark), usually done in the late evening before the dark cycle start. The ratio of male and female in the breeding tank usually is 1:2, and if the breeding tank is too small it is best not to crowd the fish tank with too many fishes (Jeanray *et al.,* 2015). The fishes also needed to be fed with generous amount of brine shrimp before putting them inside the breeding tank.

In the morning when the light cycle start, the water inside the breeding tank will be replaced with a fresh one from the system and the separator will be lift up to allow them to mate. The breeding tank will be tilt a bit to create a shore like environment inside the breeding tank as the fishes have a higher affinity to mate in that condition (Engeszer *et al.*, 2007).

The fishes will be left to spawn and it is best to avoid spawning them in a noisy environment. Usually it will take about 30 minutes to 1 hour or more for them to spawn (Spence *et al.,* 2007). After spawning, the male and female will be placed back in to their tank respectively in the system and feed right after. It is recommended not to spawn the same fish more than once a week as they need time to recover and the female usually need few days to produce new eggs (Spence *et al.*, 2007).

3.1.4 Egg collection

If there is eggs present, they will be collected from the breeding tank and put inside a petri dish with E3 (S1) solution or egg water. The eggs will be observed